

AD-A259 165



REPORT DOCUMENTATION PAGE

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2b. DECLASSIFICATION/DOWNGRADING SCHEDULE		1b. RESTRICTIVE MARKINGS	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 92-104		3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited	
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research Institute		6b. OFFICE SYMBOL (If applicable)	
7a. NAME OF MONITORING ORGANIZATION Naval Medical Command		7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, DC 20372-5120	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research & Development Command		8b. OFFICE SYMBOL (If applicable)	
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER		10. SOURCE OF FUNDING NUMBERS	
8c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, MD 20889-5044		PROGRAM ELEMENT NO. 63706N	PROJECT NO. M0095.003
		TASK NO. 1007	WORK UNIT ACCESSION NO. DN677130
11. TITLE (Include Security Classification) The protein tyrosine kinase inhibitor herbimycin A, but not genistein, specifically inhibits signal transduction by the T cell antigen receptor			
12. PERSONAL AUTHOR(S) Graber M, June CH, Samelson LE, Weiss A			
13a. TYPE OF REPORT journal article	13b. TIME COVERED FROM TO	14. DATE OF REPORT (Year, Month, Day) 1992	15. PAGE COUNT 10
16. SUPPLEMENTARY NOTATION Printed from: International Immunology 1992 Vol. 4 No.11 pp. 1201-1210			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
<div>DTIC ELECTE JAN 05 1993 S E D</div>			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Librarian.		22b. TELEPHONE (Include Area Code) (301) 295-2188	22c. OFFICE SYMBOL MRL/NMRI

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

The protein tyrosine kinase inhibitor herbimycin A, but not genistein, specifically inhibits signal transduction by the T cell antigen receptor

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Key words: genistein, herbimycin A, T cell antigen receptor

Abstract

Several lines of evidence implicate a regulatory tyrosine phosphorylation in the activation of phospholipase C (PLC) by the T cell antigen receptor (TCR). These include studies using inhibitors of protein tyrosine kinases (PTKs). In Jurkat T cells expressing the heterologous human muscarinic receptor (HM1), PLC activity can be induced by either the TCR or HM1. HM1 activates PLC via a guanine nucleotide binding protein. We have studied the selectivity of the effects of the PTK inhibitors, herbimycin A and genistein, in this system. The results indicate that these inhibitors have different mechanisms of action, and suggest that herbimycin A, but not genistein, is a specific inhibitor of PTKs in T cells. Herbimycin A markedly inhibited both the resting and induced levels of phosphotyrosine-containing proteins, including the $\gamma 1$ isozyme of PLC and the ζ chain of the TCR, and prevented activation of PLC by anti-TCR mAb. Herbimycin A did not inhibit activation of PLC by HM1. Genistein had a much less pronounced effect than herbimycin A on the appearance of tyrosine phosphoproteins. Moreover, genistein inhibited activation of PLC by both the TCR and HM1, and inhibition was only partial. Genistein was cytotoxic and markedly inhibited protein synthesis in both Jurkat cells and human peripheral lymphocytes. Herbimycin A was not cytotoxic. These findings confirm the role of a regulatory tyrosine phosphorylation in activation of PLC by the TCR. Herbimycin A was a selective inhibitor of a subclass of PTKs in Jurkat cells. In contrast, inhibition of signal transduction and later events in T cells by genistein may be due to effects other than direct inhibition of PTK activity.

Introduction

The TCR is a multimeric transmembrane structure which does not have intrinsic protein tyrosine kinase (PTK) activity (1). Engagement of the TCR initiates activation of a PTK, or cascade of PTKs, which have not definitively been identified, but may include the *src*-related PTKs *lck* (2), which associates with the T cell surface molecules CD4 and CD8 (3,4), and *fyn* (5), which co-immunoprecipitates with the TCR (6). One consequence of PTK activation by the TCR is tyrosine phosphorylation, and thus activation, of the $\gamma 1$ isozyme of phospholipase C (PLC $\gamma 1$) (7–9). A number of other intracellular substrates, including the ζ chain of the TCR (TCR ζ) and the proto-oncogene *vav* (10), are rapidly tyrosine phosphorylated following ligand binding to the TCR

(11,12). Activation of PLC $\gamma 1$ leads to the hydrolysis of phosphatidylinositol 4,5-bis-phosphate (PIP₂) to inositol 1,4,5-tris-phosphate (IP₃) and diacylglycerol, resulting in an increase in intracellular free calcium ([Ca²⁺]) and the activation of protein kinase C (PKC) respectively (13).

Several lines of evidence implicate a regulatory role for tyrosine phosphorylation in activation of PLC by the TCR, and in the subsequent expression of T cell surface molecules and IL-2 (9,14–23). The appearance of new tyrosine phosphoproteins in response to TCR stimulation precedes measurable hydrolysis of PIP₂ (15). In addition, PLC $\gamma 1$, the principal PLC activity contained in immunoprecipitated tyrosine phosphoproteins from

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Transmitting editor: L. Lanier

Received 22 April, 1992, accepted 26 June 1992

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Jurkat cells after TCR stimulation, is catalytically activated by tyrosine phosphorylation by growth factor receptors (24,25), and is tyrosine-phosphorylated following TCR stimulation in Jurkat cells and in normal T cells (9).

Further evidence for a requirement for regulatory tyrosine phosphorylation in activation of PLC by the TCR comes from studies of the effects of inhibitors of protein tyrosine phosphorylation (16,18–22). These include the benzoquinoid ansamycin antibiotic herbimycin A (16) and the isoflavone genistein (18,20,22). Herbimycin A is an *in vivo* inhibitor of *src*-related PTKs, which acts by depleting the intracellular level of enzyme (26,27). In T cells, herbimycin A reduces the immunoprecipitated activity of the *src*-related PTKs *lck* and *lyn* by >90% (16). Genistein is an *in vitro* PTK inhibitor, which may act by non-competitive inhibition of ATP hydrolysis (28).

To examine the specificity of these PTK inhibitors in T cells, we have investigated their effects on signal transduction in Jurkat cells transfected with the human muscarinic receptor type 1 (HM1) (29), which is normally expressed in neuronal and muscle cells (30). This seven transmembrane-domain receptor activates an isozyme of PLC in a PTK-independent manner, by direct interaction with a guanine nucleotide binding protein (G protein) (30–34). In this report we show that HM1 maintains its functional interaction with a G protein when expressed heterologously in Jurkat cells. In this system, herbimycin A was an effective inhibitor of TCR-initiated PTK and PLC activities, but did not inhibit activation of PLC by HM1. Inhibition of TCR, but not of HM1, activation of PLC by herbimycin A provides confirmatory evidence that a regulatory tyrosine phosphorylation, involving a member of the *src* family of PTKs, is required for activation of PLC γ 1 by the TCR. As previously reported (18), genistein also inhibited activation of PLC by the TCR. However, genistein was a relatively poor inhibitor of *in vivo* PTK activity in these cells and also inhibited PLC activation by HM1. Genistein was cytotoxic, and inhibited protein synthesis in Jurkat cells, human peripheral lymphocytes, and lectin-activated blasts. Herbimycin A was not cytotoxic and did not inhibit protein synthesis in Jurkat cells. Taken together, these results suggest that inhibition of signal transduction and later events in T cells by genistein may be due to effects other than direct inhibition of PTK activity.

Methods

Cells and reagents

Jurkat cells were maintained in RPMI 1640 medium, supplemented with 5% FCS, 5000 U/ml penicillin, 5 mg/ml streptomycin, and 200 mM glutamine (medium). The Jurkat-derived clone J-HM1-2.2 (29) expresses a functional transfected HM1. J-HM1-2.2 cells were maintained in medium with 2 mg/ml geneticin (Gibco, Grand Island, NY), and transferred to geneticin-free medium 48 h before experiments to prevent aminoglycoside-mediated inhibition of phosphoinositide hydrolysis (35). Human peripheral lymphocytes were isolated by separation on a ficoll-hypaque discontinuous gradient (Histopaque 1077, Sigma, St Louis, MO), and adherent cells were depleted by incubation on plastic tissue culture dishes. Activated lymphocyte blasts were prepared by incubating these cells with 0.1 μ g/ml phytohemagglutinin (PHA; Sigma) in medium for 48–72 h. Genistein was purchased from ICN (Cleveland, OH). Herbimycin A was

the kind gift of Dr E. Uehara. Herbimycin A and genistein were dissolved in DMSO. Genistein is poorly soluble in aqueous solutions, even when previously dissolved in DMSO. Care was taken in all experiments to insure that genistein was dissolved completely. DMSO alone, at appropriate concentration, which did not exceed 1%, was included in all controls. Additional reagents were purchased as follows; ionomycin (Calbiochem La Jolla, CA), indo-1 from (Molecular Probes Eugene, OR), carbamoyl choline chloride (carbachol), and atropine sulfate (Sigma).

Antibodies

The following mouse mAbs were used in these studies. C305 (IgM) reacts with the Jurkat TCR (36). The anti-TCR ζ mAbs 388 and 391 were the kind gifts of Dr Richard Klausner. Anti-PLC γ 1, a pool of mAbs, was the kind gift of Dr Sue Goo Rhee (7). 4G10 reacts with phosphotyrosine (37). Goat anti-mouse IgM was purchased from Zymed (San Francisco, CA).

Measurement of the affinity of the HM1 receptor for muscarinic agonist in J-HM1-2.2 cells

J-HM1-2.2 cells were disrupted by nitrogen cavitation in the following buffer: 20 mM HEPES, pH 8, 4 mM MgCl₂, 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.15 M NaCl. A membrane fraction was prepared in the following buffer (membrane buffer): 20 mM HEPES, pH 8.0, 4 mM MgCl₂, 1 mM EDTA, and 2 mM 2-mercaptoethanol, by sequential centrifugation at 2000 and 15,000 r.p.m., then homogenized and re-centrifuged at 15,000 r.p.m. Membranes were washed extensively in membrane buffer and stored at –70°C in the following buffer: 20 mM HEPES, pH 8, 2 mM MgCl₂, 1 mM EDTA, and 10% glycerol. Triplicate or quadruplicate samples of 10–30 μ g membrane protein/ml were incubated in membrane buffer with the muscarinic antagonist 1-quinuclidinyl-[phenyl-4-³H]benzylide ([³H]QNB) (Amersham, Arlington Heights, IL), 0.1 nM, a concentration previously shown to be saturating. The indicated concentrations of carbachol were added, and samples equilibrated at 37°C for 30 min, then transferred to Whatman GF/B glass fiber filters (Whatman, Maidstone, UK). Filters were washed extensively at 4°C with 5 mM Tris, pH 7.4, and 4 mM MgCl₂, placed in scintillation fluid, and ³H counted. Non-specific binding was estimated by incubation with 10 μ M atropine and was <10% in all experiments. The results are expressed as percentage inhibition of maximum binding.

Western blots of whole cell lysates and immunoprecipitation of PLC γ 1 and TCR ζ

J-HM1-2.2 cells were incubated with the 3 μ M herbimycin A or medium alone for 18 h, suspended at 10⁸ cells/ml, and stimulated for 90 s with 1:1000 C305 ascites or 500 μ M carbachol. Where indicated, 0.4 mM genistein was added 10 min before stimuli and was present throughout. Samples were lysed in ice-cold 1% NP-40 with phosphatase and protease inhibitors (38). PLC γ 1 and TCR ζ were immunoprecipitated with protein A-Sepharose beads coated with the appropriate mAb. Phosphotyrosine-containing proteins were resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose, and detected with the mAb 4G10. 4G10 was reacted with either alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad, Richmond, CA), or with ¹²⁵I-conjugated protein A

(Amersham). [125 I]protein A was quantitated by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA), using ImageQuant software.

Phosphoinositide assays

J-HM1-2.2 cells were loaded with *myo*-[3 H]inositol (Amersham) as previously described (39) and incubated for 18 h at 5×10^6 cells/ml with the indicated concentration of herbimycin A or medium alone. Triplicate samples of 10^6 cells were incubated for 20 min in 20 mM LiCl₂ and resuspended in 10 mM LiCl₂ at 4×10^6 cells/ml. Where indicated, genistein was added at the indicated concentration 10 min before stimulation and was present throughout. Samples stimulated with mAb were pre-coated at 4°C with 1:1000 C305 ascites, a concentration previously shown to be saturating. Carbachol (500 μ M) was added where indicated, and the cells centrifuged onto culture dishes coated with goat anti-mouse IgM (Zymed) and warmed to 37°C. After 30 min, reactions were stopped and cells were lysed by three alternate 5 min immersions in dry ice/ethanol and 70°C bath. Total soluble inositol phosphates were extracted by ion exchange chromatography as previously described (39).

Determination of $[Ca^{2+}]_i$

Cells were incubated for 18 h with 3 μ M herbimycin A or medium alone, loaded with the Ca^{2+} -sensitive fluor indo-1 (40), washed, and re-suspended at 5×10^6 cells/ml. Indo-1 fluorescence was measured in a Spex fluorolog II spectrofluorimeter (Spex, Trenton, NJ). The fluorimeter was calibrated for each determination by complete lysis with Triton-X 100, followed by chelation of Ca^{2+} with EGTA. Increases in $[Ca^{2+}]_i$ were calculated by the ratio method of Grynkiewicz *et al.* (40). The effects of genistein on indo-1 fluorescence could not be assessed by this fluorimetric method because solutions of genistein have substantial peaks of absorbance and of emission coinciding with those of indo-1; 220–440 and 450–480 respectively (data not shown). In order to test the effects of genistein on $[Ca^{2+}]_i$, flow cytometry was used to estimate $[Ca^{2+}]_i$ in individual cells (41). The results were analyzed for the percentage of responding cells, as determined with a histogram subtraction algorithm (Phoenix Flow Systems, San Diego, CA) (16).

Assays of cell growth

Assays to determine the effects of herbimycin A and genistein on the growth of Jurkat cells were carried out as follows. Cells were pre-incubated overnight with herbimycin A at the indicated concentration or medium alone. Cells were then suspended at 10^6 cells/ml in the indicated concentration of herbimycin A or genistein and cultured for 24 h. Cells were counted and viability determined by trypan blue exclusion.

Measurement of protein synthesis: incorporation of [35 S]methionine into total cellular protein

These assays were carried out in conditions which paralleled those used in signal transduction assays. J-HM1-2.2 cells, human peripheral lymphocytes or PHA-stimulated blasts were incubated for 18 h with the indicated concentration of herbimycin A or medium alone. Duplicate samples of 5×10^6 cells per condition were washed twice in PBS, resuspended in cysteine/methionine-free medium, incubated for 30 min at 37°C, washed in PBS, and resuspended in cysteine/methionine-free medium, 10^7 cells/ml.

[35 S]methionine (ICN, Irvine, CA), 1.0 μ Ci/sample, was added. Where indicated, genistein was added 10 min before [35 S]methionine. Cells were lysed by three alternate 5 min immersions in dry ice/ethanol and 70°C baths. Total cellular proteins was precipitated by the addition of 100 μ l 1% bovine serum albumin and 600 μ l 40% trichloroacetic acid. Pellets were washed in 10% trichloroacetic acid, and the incorporation of radiolabelled amino acids in total protein estimated. For each experiment, [35 S]methionine was added to an additional set of samples after lysis. Non-specific counts were <10% of experimental counts and were subtracted from each point.

Results

HM1 interacts directly with a G protein when transfected into Jurkat cells

In its normal cellular environment, the seven transmembrane domain HM1 activates PLC by direct interaction with a G protein (30,33). In order to investigate the mechanism of PLC activation by HM1 when transfected into the Jurkat cell line, we prepared membranes from J-HM1-2.2 cells and assayed for displacement of the muscarinic antagonist [3 H]QNB by the muscarinic agonist, carbachol. Membranes were washed free of guanine nucleotide. Under these conditions the G protein α subunit is not occupied by guanine nucleotide and the receptor which is associated with G protein is in the high affinity state for ligand. When guanine nucleotide is added it binds to the G protein α subunit, converting the G protein – receptor complex to the low affinity state for ligand. This effect is independent of the species of guanine nucleotide used (42). The decrease in affinity is measured as a shift in the dose – response curve for the displacement of the antagonist [3 H]QNB by the agonist carbachol. As shown in Fig. 1, the addition of GTP to J-HM1-2.2 membranes reduced the affinity of HM1 for carbachol, confirming

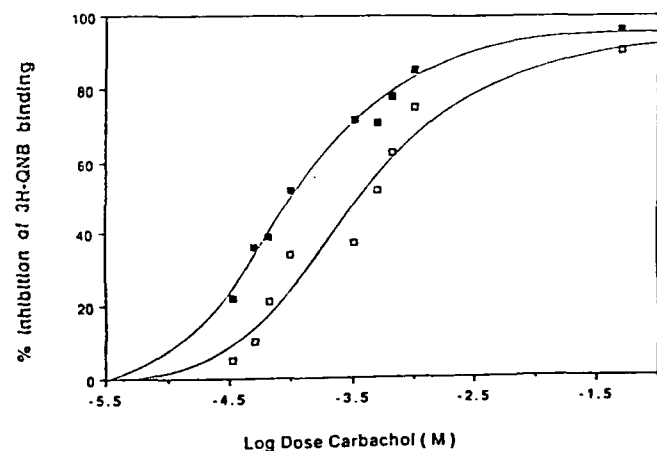


Fig. 1. Effect of GTP on the affinity of HM1 for the muscarinic agonist carbachol. Aliquots of the membrane fraction prepared from J-HM1-2.2 cells were incubated with a saturating concentration of [3 H]QNB and varying amounts of carbachol, in the presence of (□) or absence (■) of 300 μ M GTP. The fraction of 3 H counts bound to the membranes is expressed as the percentage of maximum. Each point is the mean of three determinations. A representative experiment is shown ($n = 5$).

that direct interaction of HM1 with a G protein is maintained when this receptor is expressed in Jurkat cells. Based on this evidence, and the fact that HM1 functions normally both in Jurkat cells which do not express the TCR and in TCR signalling mutants of Jurkat (29), we conclude that the TCR and HM1 regulate PLC activity by independent mechanisms in J.HM1-2.2 cells. We used the transfected HM1 as a specificity control for possible non-PTK-dependent inhibition of PLC by the PTK inhibitors in the following experiments.

Herbimycin A and genistein have differential effects on the level of basal and stimulated phosphotyrosine-containing proteins

To assess the effects of the *in vitro* PTK inhibitors herbimycin A and genistein on the induction of tyrosine phosphoproteins by

the TCR and HM1, we probed Western blots of whole cell lysates of unstimulated or stimulated J-HM1-2.2 cells, untreated or treated with inhibitor, with the anti-phosphotyrosine mAb 4G10. As previously reported (12), stimulation of untreated cells with anti-TCR mAb induced the rapid appearance of a number of new phosphotyrosine-containing proteins and an increase in the intensity of others (Fig. 2a). Carbachol induced the appearance of a tyrosine phosphoprotein of 42 kDa (lane 3). We believe this to be microtubule-associated protein-2 kinase (MAP-2 kinase), the appearance of which requires phosphorylation on both tyrosine and threonine residues (43,44). A tyrosine-phosphorylated band of similar mobility is induced by PMA in Jurkat or J-HM1-2.2 cells (38). In addition, in TCR signalling mutant variants of Jurkat (36), the tyrosine phosphorylation of p42 correlates with

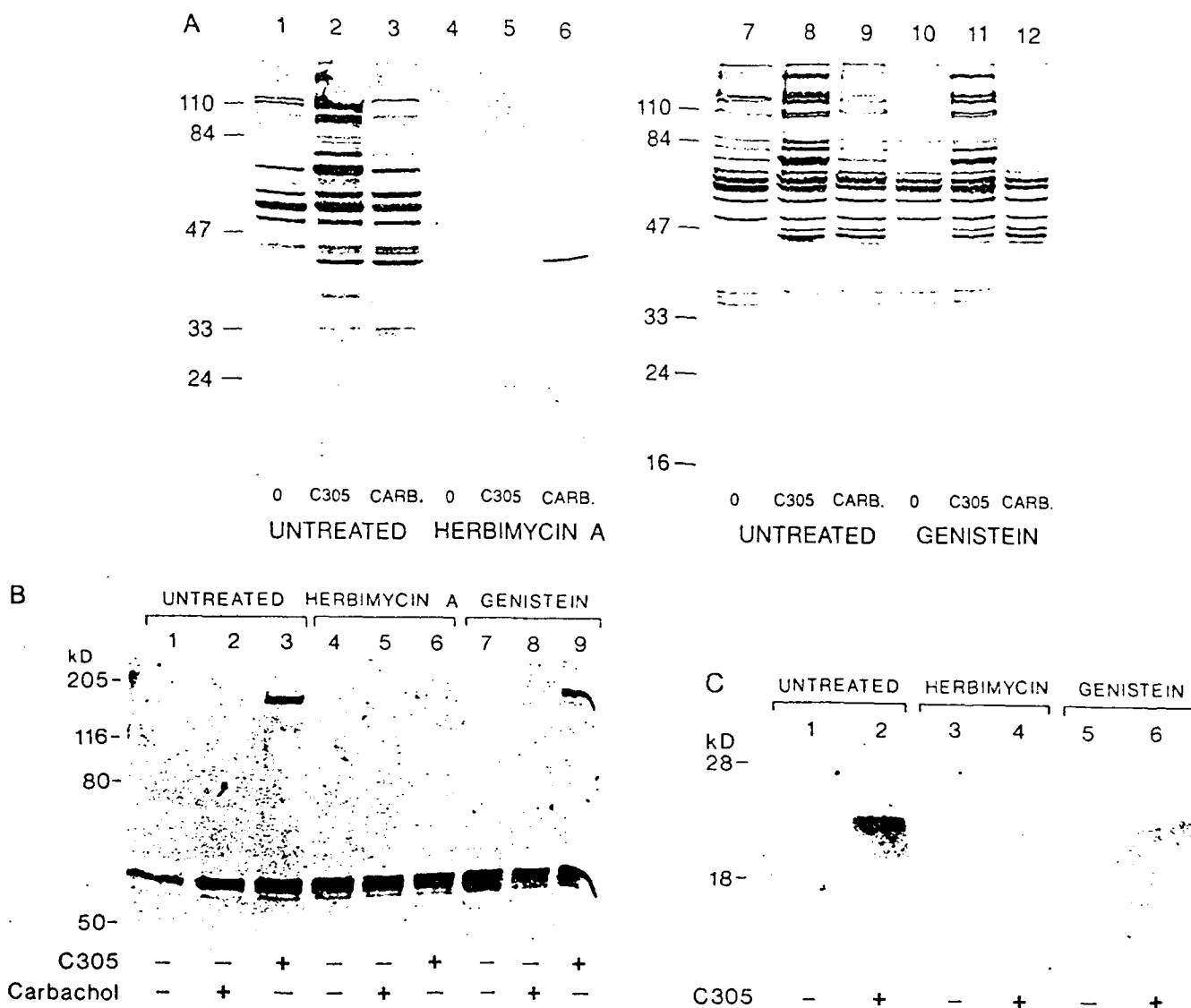


Fig. 2. Effects of herbimycin A and genistein on protein tyrosine phosphorylation in J-HM1-2.2 and Jurkat cells. (a) Cells were stimulated with anti-TCR mAb (lanes 2, 5, 8, and 11), or carbachol (lanes 3, 6, 9, and 12), following incubation in medium alone (lanes 1–3 and 7–9), 3 μ M herbimycin A (lanes 4–6), or 0.4 mM genistein (lanes 10–12). Whole cell lysates were probed with 4G10. (b) PLC γ 1 was immunoprecipitated from lysates of J-HM1-2.2 cells stimulated with C305 (lanes 3, 6, and 9), or carbachol (lanes 2, 5, and 8). Cells were pretreated with medium (lanes 1–3), herbimycin A (lanes 4–6), or genistein (lanes 7–9). (c) TCR ζ was immunoprecipitated from lysates of Jurkat cells stimulated with C305 (lanes 2, 4, and 6), and pre-treated as in (b). 4G10 was detected with [125 I]protein A.

induction of MAP2 kinase activity in *in vitro* kinase assays (Nel and A. Weiss, unpublished data).

Herbimycin A markedly reduced both the basal and TCR-stimulated level of phosphotyrosine-containing proteins, including p42, but did not substantially inhibit the induction of p42 by baclofen (Fig. 2a, lane 6). Because the appearance of p42 coincides with activation of PKC in these cells (38,44), herbimycin does not appear to inhibit either the PTK or the PLC activated by HM1. We next examined the effects of genistein on *in vivo* tyrosine phosphorylation in J-HM1-2.2 cells. Genistein at 0.4 mM, a dose which had maximal inhibitory effects on activation of PLC and protein synthesis (see below), had little detectable effect on the basal or stimulated level of the majority of cellular tyrosine phosphoproteins (Fig. 2a, lanes 7–12).

We next examined the effects of these inhibitors on tyrosine phosphorylation of two of the known substrates of the TCR-activated PTK pathway, PLC γ 1 and TCR ζ . Tyrosine phosphorylation of PLC γ 1 was virtually abolished by pre-treatment of the cells with herbimycin A (Fig. 2b). Genistein also inhibited the tyrosine phosphorylation of PLC γ 1, but to a much lesser extent than did herbimycin A. The absolute amount of cellular PLC γ 1 was not reduced by treatment with either herbimycin A or genistein (data not shown). Genistein has previously been shown to inhibit increases in the level of tyrosine phosphorylation of the TCR ζ response to TCR stimulation in peripheral T cells (18). We carried out immunoprecipitation and quantitative phosphorimager analysis of 4G10 blots of TCR ζ . Genistein inhibited the tyrosine phosphorylation of TCR ζ in Jurkat cells by 6-fold. Herbimycin inhibited tyrosine phosphorylation of this component of the TCR by 28-fold (Fig. 2c).

Herbimycin A, but not genistein, differentially inhibits activation of PLC by the TCR and HM1 in J-HM1-2.2 cells

As anticipated from inhibition of tyrosine phosphorylation of PLC γ 1, herbimycin A virtually abolished activation of phosphoinositide hydrolysis by the TCR. Inhibition of PLC activation was dose-dependent. Also in accord with the lack of inhibition of tyrosine phosphorylation of MAP-2 kinase, activation of PLC by HM1 was not inhibited by herbimycin A (Fig. 3a). In contrast, genistein was a relatively non-selective inhibitor of PLC activation in these cells. In the presence of genistein both TCR- and HM1-mediated activation of PLC were partially inhibited, in parallel, by up to 60% (Fig. 3b).

TCR- and HM1-mediated increases in $[Ca^{2+}]_i$ were differentially inhibited by herbimycin A

An increase in $[Ca^{2+}]_i$ following activation of PLC and the generation of IP $_3$ is a multi-step process involving release of Ca^{2+} from intracellular stores and flux across the plasma membrane. Fluorimetry was used to assess the response of herbimycin A-treated J-HM1-2.2 cells to stimulation with anti-TCR Ab or carbachol. Pre-treatment of J-HM1-2.2 cells with herbimycin A abolished the increase in $[Ca^{2+}]_i$ in response to an anti-TCR mAb C305 (Fig. 4). Herbimycin A had no effect on the response to the muscarinic agonist carbachol. Thus, inhibition of transmembrane signalling by herbimycin A selectively inhibits TCR- but not HM1-mediated regulation of PLC activity.

Genistein did not inhibit increases in $[Ca^{2+}]_i$ in response to stimulation of the TCR

The effects of genistein on increases in $[Ca^{2+}]_i$ could not be examined in the fluorimeter because the fluorescence spectrum

of genistein in solution coincides with that of indo-1 at 220–440 and 450–480 nm. The response of individual cells was examined by flow cytometry using the ratio method of measurement of $[Ca^{2+}]_i$. Pre-treatment with 0.4 mM genistein had no effect on the response of indo-1-loaded Jurkat cells stimulated with anti-CD3 mAb (Fig. 5). We noted increases in basal $[Ca^{2+}]_i$ in some cells following genistein pre-treatment, as well as changes in cell size as assessed by forward scatter. These effects of genistein may be attributable to cytotoxicity (data not shown, and see below).

Genistein, but not herbimycin A, inhibited protein synthesis and reduced cell viability in J-HM1-2.2 cells

Both genistein (18,45,46) and herbimycin A (16,47), have previously been reported to induce reversible cell cycle arrest in G $_0$ /G $_1$. The finding that genistein inhibited the activation of PLC by two receptors with distinct signal transduction

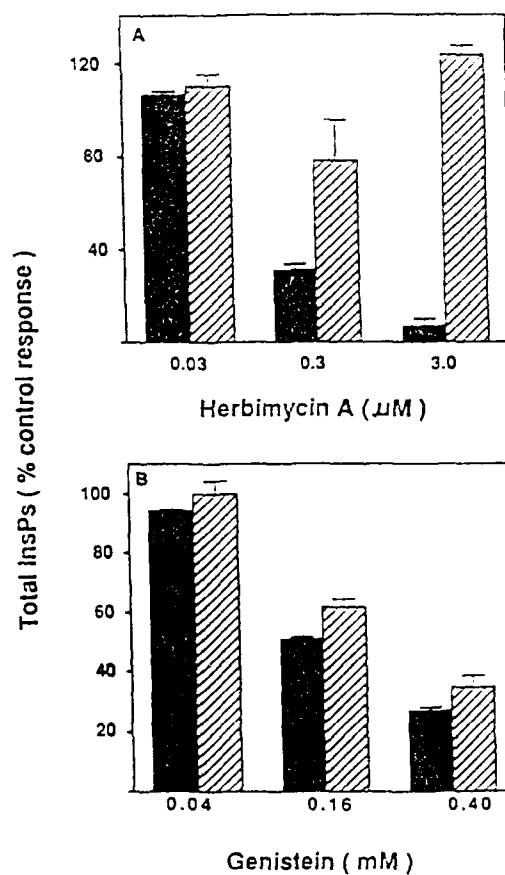


Fig. 3. Effects of herbimycin A and genistein on phosphoinositide hydrolysis in response to anti-TCR mAb and carbachol in J-HM1-2.2 cells. J-HM1-2.2 cells were incubated for 18 h in herbimycin A, at the indicated concentrations, or medium alone, and washed. Genistein was added 10 min before stimuli and was present throughout the assay. Accumulation of water-soluble inositol phosphates was measured over 30 min in the presence of LiCl $_2$. (a) Effects of herbimycin A and (b) effects of genistein on the responses to mAb C305, 1:1000 dilution of ascites (solid bars) and to 500 μM carbachol (hatched bars). Each panel is representative of at least three experiments. The SEM of each triplicate measurement was <15% in all cases. The results are expressed as the percentage of the maximal response to each agonist, obtained in the absence of the relevant inhibitor.

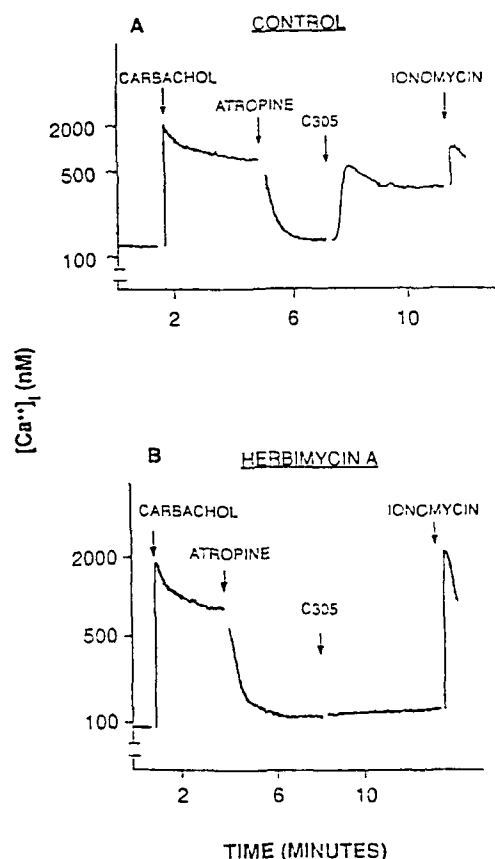


Fig. 4. The effect of herbimycin A on increases in $[Ca^{2+}]_i$ in response to anti-TCR mAb and carbachol in J-HM1-2.2 cells. J-HM1-2.2 cells were incubated for 18 h in medium alone (A) or 3 μ M herbimycin A (B), washed, and loaded with indo-1. Changes in indo-1 fluorescence were measured and $[Ca^{2+}]_i$ calculated as described in the text. Each panel is representative of two experiments.

mechanisms in the same cell, and that inhibition of both PTK and PLC activities by genistein was incomplete, suggested that genistein may have cytotoxic effects in T cells. As anticipated, the growth of J-HM1-2.2 cells was arrested by both herbimycin A, $> 0.3 \mu$ M, and by genistein, $> 0.16 \mu$ M (data not shown). However, we noted a striking difference in the effects of these reagents on cell viability and protein synthesis. Inclusion of genistein in cultures of J-HM1-2.2 cells induced dose-dependent cytotoxicity, as assessed by the ability to exclude trypan blue (Fig. 6a). The dose of genistein which maximally inhibited PIP_2 hydrolysis in signal transduction experiments reduced cell viability by 50%. Herbimycin A had no appreciable effect on exclusion of trypan blue. The ability to exclude trypan blue is a relatively insensitive means of assessing cytotoxicity. We examined the effect of genistein and herbimycin A on protein synthesis by measuring the incorporation of $[^{35}S]$ methionine into total cellular protein (Fig. 6b). Herbimycin A had no appreciable effect on protein synthesis. A 10 min pre-incubation with genistein inhibited protein synthesis, in a dose-dependent manner, by up to 80% at 0.4 mM. Inhibition was present at 2 min and was maximal at 60 min (data not shown).

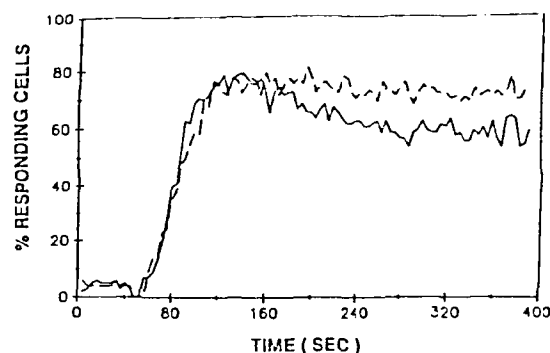


Fig. 5. The effect of genistein on anti-CD3 mAb-induced increases in $[Ca^{2+}]_i$ in Jurkat cells. Jurkat cells were loaded with indo-1 and incubated in vehicle alone (—) or genistein (---) for 10 min at 37°C. Cells were then analyzed on the flow cytometer at 200 cells/min. A baseline was obtained and the anti-CD3 mAb G19-4 (5 μ g/ml) added. The results are expressed as the percentage of cells responding with an increase in $[Ca^{2+}]_i$, > 2 SD over baseline.

Genistein inhibited protein synthesis in human peripheral lymphocytes and lymphocyte blasts

In order to exclude the possibility that genistein behaves as a non-specific inhibitor of signal transduction in Jurkat-derived cells because of a cytotoxic effect unique to Jurkat cells, we examined the effect of genistein on protein synthesis in resting human peripheral lymphocytes and lectin-activated blasts (Table 1). Pre-treatment of adherent cell-depleted human peripheral mononuclear cells with 0.4 mM genistein reduced the incorporation of $[^{35}S]$ methionine into total protein by 85%. In blast cells derived by stimulation with PHA, protein synthesis was inhibited by genistein by 47%. Genistein thus inhibited protein synthesis in both resting and activated human lymphocytes.

Discussion

A number of recent studies have used PTK inhibitors, including herbimycin A and genistein, to examine the role of tyrosine phosphorylation in signal transduction by the TCR and T cell activation. Based on the effects of PTK inhibitors on signalling by the TCR, studies have concluded that a regulatory tyrosine phosphorylation is required for activation of PLC by the TCR (16,18,19,21). However, two studies have shown that genistein incompletely inhibits activation of PLC by the TCR (20,22), suggesting that tyrosine phosphorylation may not be essential for activation of PLC by the TCR. We investigated the possibility that these divergent findings were due to differences in the specificity of *in vitro* PTK inhibitors for PTKs in intact T cells.

In order to control for possible effects other than PTK inhibition, we used the cell line J-HM1-2.2, a derivative of the T cell leukemic line Jurkat. J-HM1-2.2 expresses both the TCR and a transfected heterologous receptor, HM1. HM1 is a seven transmembrane-domain receptor, which is normally expressed in muscle and neuronal cells, but not in hematopoietic cells. Although both the TCR and HM1 both initiate PI hydrolysis in J-HM1-2.2 cells, they activate PLC by independent mechanisms, and it is likely that they activate different isozymes of PLC (48,49). Thus, inhibition

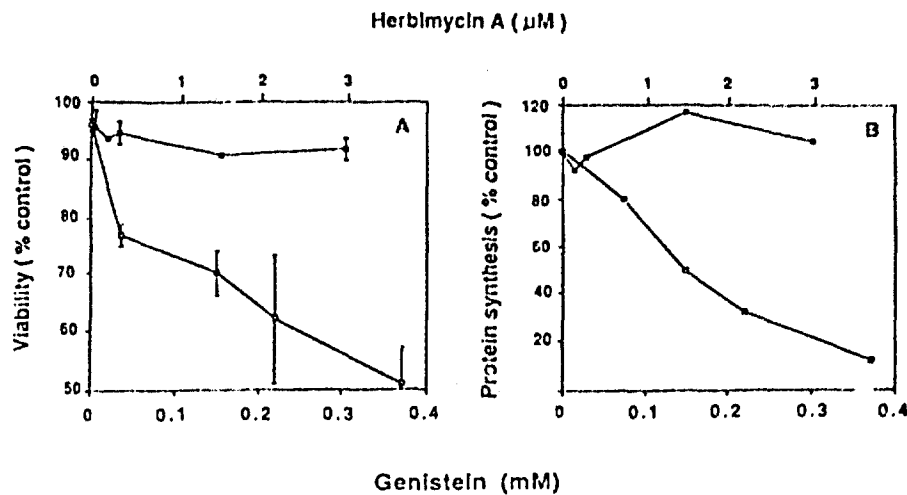


Fig. 6. The effects of herbimycin A and genistein on viability and on incorporation of [35 S]methionine into total protein in J-HM1-2.2 cells. (A) Cells (10^6 /ml) were incubated overnight (18–24 h) in medium containing the indicated concentration of herbimycin A (■) or genistein (□) and counted. The results are expressed as the percentage of cells which excluded trypan blue. Each point is the mean of at least two experiments, with duplicate determinations made for each experiment. (B) Cells (5×10^6), in duplicate, were incubated for 18 h in herbimycin A (■), at the indicated concentrations, or medium alone, then in cysteine/methionine-free medium for 30 min, and [35 S]methionine added. Genistein (□) was added 10 min before [35 S]methionine. Incorporation of radiolabelled amino acids was determined at 1 h. Each point is the mean of at least two determinations. Results are expressed as percentage incorporation of [35 S]methionine relative to untreated cells.

of signal transduction by both the TCR and HM1 by a putative specific PTK inhibitor is evidence that the inhibitor behaves non-specifically. The evidence that the TCR and HM1 activate PLC by independent mechanisms when HM1 is expressed heterologously in Jurkat cells is summarized as follows: HM1 activates PLC normally in response to carbachol when it is expressed in a Jurkat-derived cell line which lacks surface TCR, and also in Jurkat-derived signal transduction mutants which fail to activate PLC in response to anti-TCR mAb (29). We show that in J.HM1-2.2 cells, stimulation of the TCR, but not of HM1, induces tyrosine phosphorylation of PLC γ 1. Additional evidence for the independence of these pathways is that the activation of PLC by the TCR, but not by HM1 in Jurkat cells is regulated by the cell surface level of CD45 (17).

HM1 maintains its direct interaction with a G protein in J.HM1-2.2 cells, as the muscarinic agonist carbachol decreased the affinity of HM1 for its ligand. Guanine nucleotides may modulate signal transduction through the TCR via a cholera toxin-sensitive G protein (50,51). However, evidence is lacking for direct interaction of any component of the TCR with a classical G protein. It is relevant that Abraham *et al.* have demonstrated that cholera toxin acts as a PTK inhibitor in peripheral T cells, and that activation of PLC by the TCR, but not by a non-hydrolyzable analog of GTP, is sensitive to PTK inhibition by cholera toxin (21). The PLC isozyme which is activated by HM1 has not been identified. As tyrosine phosphorylation does not appear to be required, it is unlikely to be PLC γ 1. One candidate is PLC β 1, which is widely expressed, and has been shown in reconstituted membrane systems to be activated by G_q , a member of a recently-described family of G proteins (48,49,52).

In J.HM1-2.2 cells, herbimycin A inhibited signal transduction through the TCR and was selective since it did not inhibit activation of PLC by HM1. TCR-induced tyrosine phosphorylation of a subset of proteins, including PLC γ 1 and TCR ζ , was inhibited

Table 1. Effects of genistein on incorporation of [35 S]methionine into total protein in human peripheral lymphocytes and activated lymphocyte blasts

Cell	Protein synthesis (% inhibition)
J.HM1-2.2	80
Peripheral lymphocytes	85
Lymphocyte blasts	47

Results are expressed as percentage inhibition relative to untreated cells ($n = 2$).

by herbimycin A. Activation of PLC by the TCR was almost completely abolished, but HM1 induced responses were unaffected. Herbimycin A was not cytotoxic and did not inhibit protein synthesis in Jurkat cells, human peripheral lymphocytes, or activated lymphocyte blasts.

In contrast, genistein was a relatively non-specific inhibitor of PTK activity in Jurkat cells. Genistein partially inhibited activation of PLC by both the TCR and HM1. Genistein was cytotoxic, and inhibited protein synthesis in J.HM1-2.2 cells, in human peripheral lymphocytes, and in lymphocyte blasts. We conclude that herbimycin, but not genistein, is a specific inhibitor of signal transduction by the TCR.

Specific inhibition of TCR-mediated activation of PLC by herbimycin A supports previous evidence that activation of PLC by the TCR is by tyrosine phosphorylation of PLC γ 1 (7–9), and further implicates a *src* family PTK. Herbimycin A binds to the carboxy-terminus of *src*-related PTKs via sulfhydryl groups and increases their rate of degradation, reversibly depleting the steady-state level by 90–97%, (16,26,27). Several *src*-related PTKs, including *yes*, *fyn*, and *lck*, are expressed in T cells

(reviewed in 53). Both *fyn* and *lck* have been implicated in signal transduction by the TCR. Previous studies demonstrate that herbimycin A treatment of T cells results in decreased recovery of *fyn* and *lck* in immunoprecipitates, whereas the activity of the serine-threonine kinase *c-ras*, and aluminium fluoride-induced activation of PLC, are not reduced (16). Herbimycin A inhibits TCR-mediated early and late activation events, but does not inhibit increases in IL-2R and IL-2 induced by the combination of calcium ionophore and phorbol ester (16). This finding confirms that the PTK inhibited by herbimycin in T cells is proximal to PKC and likely to be a *src* family member.

Genistein has previously been shown to inhibit the TCR-induced tyrosine phosphorylation of TCR ζ in human peripheral lymphocytes (18). Norton *et al.* (22) have also shown that genistein inhibits the appearance of a number of tyrosine phosphoproteins on stimulation of peripheral T cells with antigen. Genistein inhibits production of IL-2 in response to TCR stimulation; however, genistein also inhibits IL-2 production in response to the combination of phorbol ester and calcium ionophore, stimuli which by-pass the requirement for activation of a PTK by the TCR (18,20). This finding suggests that genistein inhibits a step in T cell activation distal to the activation of PLC.

In this study genistein inhibited PLC activation through both the TCR and HM1 but had only a minor effect on the pattern and intensity of phosphotyrosine-containing bands stimulated by either anti-TCR mAb or carbachol in J.HM1-2.2 cells. Tyrosine phosphorylation of PLC γ 1, which was virtually abolished by herbimycin A, was inhibited much less by genistein. In agreement with the findings of Mustelin *et al.* (18), the level of tyrosine phosphorylation of TCR ζ was decreased by genistein, but was significantly less than that seen with herbimycin A. In this study and previous studies (54,55), a relatively small proportion of cellular TCR ζ is tyrosine phosphorylated in response to TCR engagement. Quantitation of TCR ζ phosphorylation is also complicated by the appearance of multiple phosphorylated forms with differing mobility on SDS-PAGE. Inhibition by genistein of tyrosine phosphorylation of TCR ζ could be interpreted to show that genistein inhibits a PTK other than that which phosphorylates PLC γ 1. Alternatively, the enzyme may be the same, but non-competitive inhibition of ATP by genistein may inhibit tyrosine phosphorylation of these substrates differentially (28; and see below). Alternatively, genistein may influence the function of a phosphatase or other intermediary molecule which is required in the tyrosine phosphorylation of TCR ζ , but not of PLC γ 1.

Previous studies have shown that genistein inhibits the *in vitro* PTK activity of the epidermal growth factor receptor (EGFR), and of *src*, *gag-fes* (28), and *lck* (20). However, effects of genistein which may not be related to inhibition of PTK activity include inhibition of serine-threonine kinase activity (56), reversal of transformation by the guanine nucleotide-binding protein *ras* (45), inhibition of DNA topoisomerase activity (45,46,57,58), and inhibition of receptor binding of thromboxane A₂ analogs (59). The specificity of genistein may also vary between tissues (56,60-64). Akiyama has proposed that the mechanism of action of genistein may be as a non-competitive inhibitor of ATP hydrolysis (28). This may account for the variability of PTK inhibition by genistein. Inhibition of ATP hydrolysis may also explain the cytotoxicity of genistein for T cells in this study, and that reported other cell types (56,57).

In summary, herbimycin A, which down-regulates *src*-related

PTKs, specifically inhibits signal transduction by the TCR. This supports the model whereby ligand binding to the TCR directly or indirectly activates an as yet-unidentified *src*-related PTK which in turn tyrosine phosphorylates and activates PLC γ 1. The *in vitro* PTK inhibitor genistein is a relatively poor inhibitor of PTK activity in intact T cells and has non-selective effects unrelated to PTK inhibition. These findings highlight some of the potential limitations of studies using inhibitors to examine the role of tyrosine phosphorylation in signal transduction, growth, and differentiation.

Acknowledgements

We thank Gary Koretzky and James Fraser for helpful comments during these studies, and critical review of the manuscript. This work was supported in part by an NIH grant to A.W. M.G. is supported by NIH grant K 11 DK01945-01.

Abbreviations

[Ca ²⁺] _i	intracellular free calcium
EGFR	epidermal growth factor receptor
G protein	guanine nucleotide-binding protein
HM1	human muscarinic receptor type 1
[³ H]QNB	1-quinuclidinyl[phenyl-4- ³ H]benzilate
IP ₃	inositol 1,4,5-tris-phosphate
MAP-2	microtubule associated protein-2
PHA	phytohemagglutinin
PIP ₂	phosphatidylinositol 4,5-bis-phosphate
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinase

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